Effect of Subchronic Feeding of Sumithion, Sumioxon and p-Nitrocresol on Rat Hepatic Oxidative Phosphorylation and Mixed Function Oxidases

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6. スミチオン，スミオキソン，*p*-ニトロクレゾールのラット亜慢性摂食時における肝臓諸酵素におよぼす影響

細川俊治，宮本純之（住友化学工業株式会社農薬事業部研究部） 50. 1. 16 受理

ウイスター系ラットに対しスミオチン 150 ppm およびその代謝物であるスミオキソン 50 ppm, *p*-ニトロクレゾール 1500, 500ppm を飼料中に混ぜ6カ月もしくは9カ月摂食させて肝ミトコンドリア酸化的リン酸化およびミクロソーム薬物代謝系諸酵素に対する影響をしらべた．ミトコンドリアの呼吸調節率および ATPase 活性はいずれの投与群も正常であり，ADP：O 比はスミオキソン投与群の雄と *p*-ニトロクレゾール 1500ppm 群の雌においてわずかに低下していた．ミクロソーム薬物代謝系のうちアニリン水酸化酵素，アミノピリン脱メチル酵素活性．チトクロム *p*-450および $NADPH_2$ 還元酵素活性については，スミオキソンを投与した雄でアニリン水酸化酵素活性がやや上昇していたのを除き各投与群とも顕著な変化は認められなかった．この結果によればかなり大量のスミチオンの連続投与によっても，肝ミトコンドリアの酸化的リン酸化やミクロソームの薬物代謝酵素は影響をうけないと推定される．

Introduction

As reported elsewhere[1], dietary administration of Sumithion (150 ppm) and its two toxicologically significant metabolites, Sumioxon (50 ppm) and *p*-nitrocresol (1,500 ppm) to rats for consecutive 6 months caused no appreciable adverse effects on liver weight, as well as on liver structure (histopathology) and function (clinical biochemistry). However, since many foreign compounds have recently been known to induce liver microsomal mixed function oxidases system[2-5] or act as inhibitor of mitochondrial oxidative phosphorylation[6,7] and some organophosphorus compounds and various types of phenol affect the drug metabolizing enzyme[8,9] or the mitochondrial respiration system[10,11], the subchronic effects of Sumithion, Sumioxon and *p*-nitrocresol on the hepatic oxidative phosphorylation and mixed function oxidases were investigated after these compounds had been consecutively administered for 6 or 9 months.

Materials and Methods

Chemicals: The following compounds were studied: Sumithion [*O,O*-Dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate] manufactured by Sumitomo Chemical Co., lot No. 417, purity 97.2 %, Sumioxon [*O,O*-Dimethyl *O*-(3-methyl-4-nitrophenyl) phosphate] purity ＞99%, *p*-nitrocresol (3-methyl-4-nitrophenol) purity ＞99.5%, synthesized in this laboratory.

Treatment of animals: Six week old male and female wistar strain rats were housed in individual cages and kept at 24±1°C. All animals were fed *ad libitum* for 6 months or partly for 9 months and had free access to water. The control group received a powdered diet (Nihon Crea CE-2) and the test group received powdered diet containing 150 ppm Sumithion, 50 ppm Sumioxon or 1,500 ppm *p*-nitrocresol[1]. After feeding period, the test group fed a control diet for 3 days and starved for 12 hours before the following experiment.

Liver fractionation: Test animals were decapitated and the livers were immediately homogenized in 10 vol. of cold buffered solution containing 0.25M sucrose, 5mM tris-HCl and 0.1mM EDTA (pH 7.5), using a Teflon-glass homogenizer. After the homogenates were centrifuged at 900g for 10 min, mitochondria fractions were collected at the 8000g pellet. The liver microsomes and the

soluble fraction were prepared by centrifuging the 8,000g supernatant at 105,000g for 60 min. The mitochondrial pellet was washed once with 0.25M sucrose solution and the microsomal pellet was washed once with 1.15 per cent KCl solution. Then these pellets were resuspended in the original volume of the buffered solution mentioned above.

Respiration and oxidative phosphorylation: Oxygen utilization, ADP to oxygen ratios and respiratory control indices were measured in an oxygraph (Kyusui kagaku Co.) with a galvanic type oxygen electrode[12,13]. The reaction mixture contained: sucrose, 0.25M; Tris buffer, 0.01M; phosphate buffer, 0.01M; KCl, 0.01M; MgCl, 2mM; EDTA, 0.2mM; succinate, 4.2mM; ADP, 0.35mM and 10mg of mitochondrial protein. The final volume of the reaction mixture in the oxygraph chamber was 2.4 m*l* at pH 7.4 and 25°C. The oxygen uptake was calculated from the change in oxygen tension measured polarographically. Fig. 1. shows the typical oxygen consumption pattern employing succinate as substrate. The oxygen uptake by the mitochondria in the chamber was calculated from the oxygen solubility of solutions equilibrated with room air at 25°C and the factor of 245μM oxygen in the chamber was utilized for calculation. The respiratory control ratio was calculated as

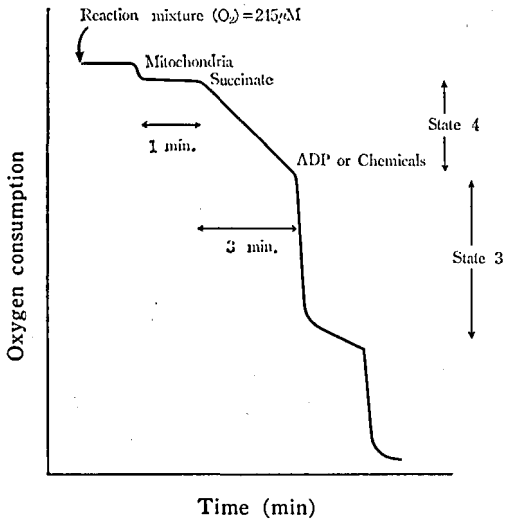

Fig. 1. Oxygen consumption pattern of succinate oxidation of rat liver mitochondria.

defined by Chance and Williams by dividing the rate of oxygen uptake during state 3 and the known quantity of ADP added[14] (Fig. 1).

The stimulation of the rate of oxygen uptake during state 4 respiration by Sumithion, Sumioxon and *p*-nitrocresol was measured in vitro. After the reaction mixture mentioned above was incubated for 1 min, oxygen consumption rate was recorded for about 3~4 min and the change of the rate of oxygen uptake was measured by the addition of the chemicals. (Fig. 1). All the chemicals were dissolved in acetone. Final concentration of acetone was about 1%.

ATPase activity: Mitochondrial ATPase was measured in a medium containing 0.05M Tris-HCl buffer (pH 7.5), 0.03M KCl, 0.005M $MgCl_2$, 0.005M ATP and 1~2 mg mitochondria protein in a total volume of 1.0 m*l*. The reaction mixture was incubated at 25°C. Inorganic phosphate was measured by the method of Fiske and Subbarow[15]. Protein content was determined by the Lowry method[16].

Microsomal enzyme assays: Demethylation of aminopyrine was determined by measuring the amount of formaldehyde formed[17]. The reaction mixture contained: phosphate buffer, 0.05M (pH 7.5); G-6-P, 0.01M; $MgCl_2$, 0.005M; NADP, 0.5mM; G-6-P dehydrogenase, 50 unit; aminopyrine, 0.004M and the specified volume of 8,000g supernatant (usually 2 to 3 m*l*) in a total volume of 5.0 m*l*. The reaction mixture was incubated at 37°C. Aromatic hydoxylation of aniline was determined by measuring *p*-aminophenol formed from aniline as substrate[18]. The same incubation condition as aminopyrine demethylation was used.

Cytochrome P-450: Cytochrome *p*-450 was determined by CO-difference spectra between absorption at 450 and 490nm after reducing by hydrosulfite[19].

$NADPH_2$-cytochrome C reductase: 8,000g supernatant was used for the estimation of $NADPH_2$-cytochrome C reductase activity in a medium containing 0.33M phosphate buffer (pH 7.6), 0.042mM $NADPH_2$, 0.001M KCl and 0.05mM oxidized cytochrome C in a total volume of 3.0 m*l*. The reaction mixture was incubated at 30°C and the increase of absorption at 550nm was measured by an automatic recording spectrophoto-

meter[20].

Results and Discussions

Effect on body and liver weights: Table 1 shows the effect of dietary Sumithion, Sumioxon and *p*-nitrocresol on body weight and liver weight. A significant increase of body weight as well as liver weight was obsevred only at the group of males fed 1,500 ppm *p*-nitrocresol. In the lower dosage group (500 ppm) the difference was not found. No difference was observed in their liver/body weight ratio in any groups.

Effect on mitochondrial respiration and oxidative phosphorylation: The effect of dietary Sumithion, Sumioxon and *p*-nitrocresol on mitochondrial ATPase and protein (Table 2), mitochondrial respiration system (Table 3) was examined. Normal mitochondrial respiration is controlled by inorganic phosphate and ADP, and oxidation of substrate is stimulated by adding ADP (state 3). This respiratory control ratio is an index of the integrity of the mitochondria. Fresh, tightly coupled, well prepared mitochondria have high control ratios[14]. Mitochondrial ATPase activity is known to be elevated with the injury of mitochondria and or in the presence of uncoupler. In the present experiments respiratory control ratio was about 4~6 enough to show the integrity of mitochondria in any groups. Similarly ATPase activity was normal and no difference was found between the test groups and the control.

Slight decrease of ADP: O ratios were observed at the group of males fed 50 ppm Sumioxon and females of 1,500 ppm *p*-nitrocresol. But difference

Table. 1. The effect of dietary Sumithion, Sumioxon and *p*-nitrocresol on body weight and liver weight of rats.

Sex	Chemicals	Dose, ppm	Month	Body weight (g)	Liver weight (g)	Index (%)
Male	Control	0	6	409±40.2	10.9 ±1.49	2.69±0.480
	Control	0	9	487± 6.16	11.8 ±0.382	2.42±0.101
	Sumithion	150	6	459±17.1	11.1 ±0.292	2.42±0.110
	Sumioxon	50	6	424±19.9	9.67±0.531	2.29±0.071
	Sumioxon	50	9	458±30.3	10.7 ±0.561	2.34±0.220
	p-nitrocresol	1500	6	547±62.3	13.2 ±0.471	2.43±0.197
	p-nitrocresol	500	9	494±61.5	12.5 ±3.74	2.51±0.090
Female	Control	0	6	261± 5.72	6.31±0.097	2.42±0.097
	Sumithion	150	6	234±19.3	6.61±0.316	2.87±0.228
	Sumioxon	50	6	296±19.7	7.29±0.925	2.46±0.152
	p-nitrocresol	1500	6	273±28.8	7.24±0.681	2.67±0.164

Mean ± S.D. (n=4 for all groups)

Table. 2. The effect on mitochondrial ATPase and protein treated with Sumithion, Sumioxon and *p*-nitrocresol.

Sex	Dose (ppm)	ATPase moles Pi/1hr/g liver	Protein mg/g liver
Male	Control	59.9±1.64	25.0±5.44
	Sumithion (150)	56.4±1.90	25.1±2.36
	Sumioxon (50)	59.0±9.39	20.2±2.61
	p-nitrocresol (1500)	47.9±5.78	15.2±1.34
Female	Control	82.8±13.8	23.6±2.01
	Sumithion (150)	85.9±5.53	20.8±2.59
	Sumioxon (50)	98.1±35.9	21.6±2.47
	p-nitrocresol (1500)	68.2±14.5	20.2±1.28

mean ± S.D. (n=4 for all group)

Table. 3. The effect on mitochondrial respiration.

Sex	Dose (ppm)	state 4 rate	state 3 rate	O_2 consumption at state 3	Respiratory control ratio	ADP : O ratio
		m moles O_2/ml/min/mg prot.		atom O/ml		
Male	Control	14.7±3.57	59.2±8.46	0.186±0.017	4.11±0.585	1.90±0.173
	Sumithion (150)	14.3±2.48	68.6±6.38	0.184±0.017	4.87±0.356	1.92±0.168
	Sumioxon*(50)	12.3±1.21	45.9±9.86	0.208±0.010	3.75±0.750	1.69±0.089
	p-nitrocresol*(500)	12.1±1.18	49.0±13.7	0.199±0.014	4.01±0.669	1.77±0.100
Female	Control	12.1±1.35	64.4±8.75	0.201±0.039	5.42±0.930	1.80±0.326
	Sumithion (150)	12.8±0.457	77.0±6.71	0.192±0.008	6.04±0.430	1.83±0.073
	Sumioxon (50)	10.7±2.80	47.9±8.46	0.189±0.024	4.53±0.336	1.88±0.251
	p-nitrocresol*(1500)	14.3±2.07	57.4±13.7	0.217±0.003	4.03±0.675	1.62±0.027

* 9 month feeding
Mean ± S.D. (n=4 for all groups)

in these results cannot be regarded as statistically significant ($p<0.05$) by student *t*-test. From these results, mitochondrial respiration system seems to be not affected remarkably by the administration of these chemicals in vivo. The possibility of the uncoupling activity of Sumithion, Sumioxon and *p*-nitrocresol was investigated in vitro by measuring the stimulation of the rate of oxygen uptake during state 4 respiration (Table 4). The stimulation index at 10^{-3}M final concentration was 1.5 for Sumithion, 1.8 for Sumioxon and 1.5 for *p*-nitrocresol, whereas 2,4-dinitrophenol increased 11.4 fold. At 2×10^{-5}M of Sumithion, Sumioxon or *p*-nitrocresol, the stimulatory effects were not observed at all, while 2,4-dinitrophenol showed marked effects. Matsuda et al.[10] reported that 10^{-2}M Sumithion and 10^{-2} or 10^{-3}M *p*-nitrocresol inhibited rat liver oxidative phosphorylation in vitro. However, these effects in vitro

Table 4. The stimulation of the rate of oxygen uptake during state 4 respiration.

Chemicals	Stimulation index	
	Final conc.	
	1×10^{-3}	2×10^{-5}
Untreated	1	1
Sumithion	1.5	1
Sumioxon	1.8	1
p-nitrocresol	1.5	1
2,4-dinitrophenol	11.4	3.3

seems to be found only at such a high concentration and rapid elimination of Sumithion and its major activation or degradation product, Sumioxon and *p*-nitrocresol, in the metabolic studies[21-30] might ensure the concentrations of these active metabolites far less than those causing inhibition on the mitochondrial enzyme activity.

Effect on microsomal mixed function oxidases:

The activities of hepatic microsomal mixed

Table 5. The effect on hepatic drug metabolizing enzyme treated for 6 months.

Sex	Dose (ppm)	Aminopyrine demethylase moles/1hr/g	Aniline hydroxylase moles/1hr/g	Cyt P 450 m moles/g	$NADPH_2$-cyt C reductase moles/min/g	Ms protein mg/g liver
Male	Control	8.18±2.18	1.08±0.266	28.7±0.770	4.44±0.850	35.5±6.79
	Sumithion (150)	6.72±1.21	1.39±0.265	23.0±0.919	3.49±0.490	37.8±3.30
	Sumioxon (50)	8.09±1.79	2.11±0.308	21.0±0.757	4.89±0.683	33.4±6.00
	p-nitrocresol (1500)	6.15±0.468	1.28±0.327	24.1±1.91	3.93±0.358	30.1±2.06
Female	Control	5.13±0.476	1.55±0.470	13.9±1.64	2.06±0.480*	21.2±2.10
	Sumithion (150)	5.56±0.302	1.74±0.318	14.6±1.84	2.04±0.064*	19.0±3.08
	Sumioxon (50)	4.06±0.714	1.46±0.407	12.7±1.62	3.03±0.302	20.8±4.15
	p-nitrocresol (1500)	4.16±0.807	1.31±0.248	14.4±1.51	2.90±0.209	20.0±0.50

* 9 month feeding
Mean ± S.D. (n=4 for all groups)

function oxidases were checked as to the demethylation of aminopyrine and aromatic hydroxylation of aniline. Cytochrome *P*-450 content and $NADPH_2$-cytochrome C reductase activity were measured also as the important constituents. As shown in Table 5, there were no significant changes except for the aniline hydroxylase activity at the group of male fed 50 ppm Sumioxon. Paraoxon enchances or inhibits the aniline hydroxylation in vivo and in vitro[8,21]. Sumioxon also inhibited aniline hydroxylation in vivo and in vitro[9]. This phenomenon seems to be much complicated and dependent on the concentration. But by the fact that the residue of Sumioxon was not detected in mammalian liver except under exaggerated trials, there seems to be no possibility of the adverse effect on microsomal systems. Similarly to the case of mitochondrial respiration, the dietary administration of Sumithion for consecutive 6 months or more appears to have no appreciable irreversible effects on these hepatic microsomal mixed function oxidases.

Summary

Subchronic effects on the rat hepatic oxidative phosphorylation and mixed function oxidases after feeding of 150 ppm Sumithion, 50 ppm Sumioxon and 1500 or 500 ppm *p*-nitrocresol in diet for 6 or 9 month were examined. A slight decrease of ADP: O ratios was observed by Sumioxon (males) and by 1500 ppm of *p*-nitrocresol (females). Respiratory control ratio and ATPase activity was normal and no differences was found between test groups and the control. The activities of aminopyrine demethylase and $NADPH_2$-cytochrome C reductase and cytochrome *P*-450 content showed no significant change in any group, but enhancement of aniline hydroxylase activity was observed in males given Sumioxon. Sumithion caused no effect on these parameters.

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Subchronic Toxicity Studies of Sumithion, Sumioxon and *p*-Nitrocresol in Rats and 92 Week Feeding Study of Sumithion with Special Reference to Change of Cholinesterase Activity. Tadaomi KADOTA, Hiroyuki KOHDA and Junshi MIYAMOTO (Research Department, Pesticides Division Sumitomo Chemical Co., Ltd., Takarazuka, Hyogo, Japan) Received Jan. 16, 1975. *Botyu-Kagaku* 40, 38, 1975.

7. スミチオン，スミオキソン，*p*-ニトロクレゾールのラットにおける亜慢性毒性およびスミチオン92週摂食によるコリンエステラーゼの変動

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有機リン殺虫剤スミチオンおよびその代謝産物の亜慢性毒性を明らかにするため最高スミチオン150ppm，スミオキソン 50ppm，*p*-ニトロクレゾール 1,500ppm を含む飼料をそれぞれ6カ月間ラットに摂食させ，体重測定，摂食・摂水量測定，血液検査，臨床生化学検査，尿検査，臓器重量測定，病理組織学的検査を実施した．

コリンエステラーゼ活性を除き，各検査項目についてこれらの化合物の投与に起因すると思われる異常は見出されなかった．血漿，血球，脳コリンエステラーゼはスミチオン，スミオキソンの投与量に相関して阻害されており，スミチオンは最低の 10ppm でも雌血漿コリンエステラーゼを有意に阻害したところから．さらに92週に及ぶ追加摂食実験を行ない経時的に血液コリンエステラーゼ活性を測定した．この条件下でスミチオンの無影響量は飼料中 5ppm，体重換算 0.27mg/kg/day であった．

一方スミオキソンの無影響量は，6カ月摂食後で飼料中 5ppm であった．

Introduction

Sumithion® or *O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate is an organophosphorus insecticide now widely used to control various plant pests and insects of medical importance. The metabolic studies in mammals[1-6] revealed that orally administered radioactive Sumithion was easily absorbed from the gastrointestinal tract and distributed into various tissues. Sumithion was confirmed to be oxidized into the active metabolite Sumioxon, *O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorate in the animal both in vivo and in vitro. Both compounds were decomposed in animal body and the radioactivity was eliminated rapidly and completely, majorly into urine. The major degradation products in urine were desmethylsumithion, desmethylsumioxon, dimethylphosphorothioic acid, dimethylphosphoric acid, *p*-nitrocresol (3-methyl-4-nitrophenol) and its conjugates. Plant metabolism proceeds in essentially the similar manner[7-9]; a trace amount of Sumioxon and *p*-nitrocresol, free and bound with glucose, were found.

Although it has already been demonstrated that residue of Sumithion and its metabolites in various harvested crops is generally low[7-13], it is necessary to assess the chronic toxicity of Sumithion and other possibly toxicologically significant metabolites in humans. In this study, therefore, Sumioxon and *p*-nitrocresol as well as Sumithion were fed to rats for consecutive 6 months to examine the subchronic effects on various physiological parameters. A supplementary study was also carried out with special reference to the change of cholinesterase activity